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DNA Base Composition of Actinomyces and Related Anaerobic Diphtheroids

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DNA BASE COMPOSITION OF ACTINOMYCES AND RELATED ANAEROBIC DIPHTHEROIDS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

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INTRODUCTION

The taxonomy of the anaerobic bacteria known as diphtheroids is a subject of study by the Subgroup on Taxonomy of Microaerophilic Actinomycetes, an authorized Subgroup of the Subcommittee on Taxonomy of the Actinomycetales of the International Committee on Nomenclature of Bacteria, International Association of Microbiological Societies. Dr. Gordon Robertstad, a member of this committee, has stimulated a series of studies on these organisms. At present, the species differentiation coupled with genus differentiation among anaerobic diphtheroids is in a state of flux. Tests which were previously thought to be indisputable are now not entirely reliable. Fermentation reactions, catalase tests, serology, and cell wall analyses are not sufficient in themselves as criteria for identification. If it is necessary to perform every known biochemical, serological, metabolic, and nutritional test to identify any isolate, then the practical identification with a minimum of time and materials is not feasible. Ideally, differentiation via one or two tests only is the goal of the taxonomist and diagnostician, as well as the researcher.

As an approach to the above problem, deoxyribonucleic acid (DNA) base ratios are now becoming a promising tool in separating organisms; that is, DNA base ratios along with DNA homologies. DNA base ratios are expressed as mole-percent guanine + cytosine (the four DNA bases are adenine, thymine, guanine, and cytosine) obtained directly by chemical means or indirectly by physical means. DNA homologies are assessed by measuring hybrid formation between single-stranded DNA's of diverse origin. Taxonomy based on properties of the DNA molecule offers a more direct mode of classification. The phenotypic properties of an organism may be more apparent to the observer,

but more subject to environmental changes. For example, cell wall analyses may change with the media used to cultivate the organisms. The genotype of the cell, as present in its DNA genome, is not affected thus; rather, it is stable until the factors of mutation and base analogs enter the picture.

The author has attempted the nucleic acid (base ratio) study in order to determine the base composition of the Actinomyces species, as well as of fourteen anaerobic diphtheroids isolated by Biever (1967); four other genera, Corynebacterium sp., Rothia sp., Ramibacterium sp., and Odontomyces sp., will also be represented. The possibility of a new species, Actinomyces suis, is so strong that a definite criterion for classification and identification must be set up. The base ratios obtained would facilitate classification of Actinomyces and related organisms, as well as assist identification of fresh isolates.

Previous attempts to determine DNA base ratios of the Actinomyces were thwarted by the resistance of many of the Actinomyces sp. to lysis and subsequent isolation of native DNA in sufficient quantities for base analysis (Smith, 1963). Thus, the investigation of methods for successful DNA extraction is made part of this study, in terms of which organisms can be lysed under a given set of conditions. The characteristics of the cell walls of the Actinomyces necessitate the selection of a combination of enzyme (s) and mechanical methods for lysis.

LITERATURE REVIEW

Review of the Genus Actinomyces and Related Diphtheroids

Organisms of the genus Actinomyces are classified in the order Actinomycetales and are characterized as having a true mycelium with vegetative mycelium fragments in bacillary or coccoid elements. Within the family Actinomycetaceae, the Actinomyces are defined as anaerobic or microaerophilic, non acid-fast organisms.

Most of the members of the genus Actinomyces are pathogenic (Waksman, 1961) and have been isolated from granules in the pus of morbid tissues of human and animal disease known as actinomycosis.

Actinomyces bovis is responsible for most cases of lumpy jaw in cattle and A. israelii causes most typical infections in man. A. bovis Harz, the first authentic Actinomyces sp. described as a causative agent of disease, was isolated by Harz in 1877. The particular strains of this and the other organisms were isolated from various human and animal lesions.

A. discofoliatus, first identified in 1932 by Grüter, is normally found in lachrymal concretions and human actinomycotic lesions.

A. israelii was first isolated by Wolff and Israel in 1891. It inhabits its dental caries, tonsils, and natural cavities of man and animals. It is the chief etiological agent of human actinomycosis.

A. naeslundii was first described by Thompson and Lovestedt (1951). It is considered to be a saprophyte in the human mouth.

A. odontolyticus was first described by Batty in 1958. It is found in human saliva and in deep dental caries.

Buchanan and Pine (1962) described another agent of actinomycosis,

A. proprionicus, which was isolated from human lachrymal canaliculitis.

In 1965, Georg et al. described a new pathogenic, anaerobic Actinomyces sp., A. eriksonii, which was named after Dr. Dagny Erikson whose classic studies on morphology and physiology of the anaerobic Actinomyces laid the groundwork for these and related organisms. Strains were isolated from pleural fluid and lung abscesses.

A. parabifidus, formerly Lactobacillus bifidus II was originally described by Weiss and Rettger in 1938. In 1956, Pine and Howell compared the physiological and biochemical characteristics of L. bifidus with Actinomyces spp. and found this organism to fit into no other genus than the Actinomyces. The relationship was further emphasized by similarity of cell wall composition.

In 1965, Howell et al. described a new genus and species as isolated from peridontal plaque in hamsters. This was designated as Odontomyces viscosus. This "hamster organism" belongs in the family Actinomycetaceae. However, it could not fit into Nocardia, and has since been placed in the genus Actinomyces (Slack, 1968).

Actinomyces dentocariosus was first isolated from carious dentine in humans by Onishi in 1949. The organism was then inserted into the Nocardia sp., and then later reclassified as Rothia dentocariosus by Georg and Brown (1967) in the family Actinomycetaceae.

Involvement of corynebacteria in actinomycosis was first described by Vawter in 1931. He successfully isolated a form of Actinomyces sp. from lesions in swine, which resembled Corynebacterium pyogenes in morphology and Gram stain reaction.

Slack and Grencser (1966) showed some serological relationship between Actinomyces spp. and Corynebacterium acnes by gel diffusion tests.

Cummins and Harris (1955) found that results of cell wall analyses indicated that Corynebacterium pyogenes and C. haemolyticum do not fit into the same category as the other corynebacteria, but do suggest a relationship with Actinomyces.

Biever (1967) isolated a series of unknown anaerobic diphtheroids from swine and bovine abscesses, which were characterized by biochemical and physiological tests including fermentation reactions of carbohydrates, catalase tests, and some serological grouping. His findings strongly suggested that certain isolates resembled Actinomyces suis. Urevig (1968) further characterized the known Actinomyces spp., as well as Biever's unknown isolates by serology. Scheetz (1969) used cell wall analyses to group the same knowns and unknowns; groupings of the unknown isolates by the above three workers basically agree, but significant differences were noted in the regrouping of the isolates (Table 5).

Deoxyribonucleic acid as the Genetic Material

Deoxyribonucleic acid (DNA) has been found to be the common denominator for heredity in both the animal and plant world. Thus, one must ultimately return to this substance for the genetic classification of characteristics of any species. In 1944, Avery, Macleod, and McCarty with in vitro experiments on pneumococcus, showed DNA definitely to be the genetic material.

In 1953, Pauling postulated a helical structure for proteins and also for polynucleotides. Based on this work and on the work of Wilkins and of Chargaff, Watson and Crick (1953) proposed the double helix model for DNA, in which each strand is composed of a sequence of deoxyribose units linked together by phosphates in a diester linkage. Each deoxyribose is connected by a glycosidic linkage to one of the four nitrogenous bases: adenine, guanine, cytosine, or thymine. The two DNA strands are interconnected by hydrogen bonds between the bases. The molecular configurations of the components are such that each adenine on one strand is connected by two hydrogen bonds to a thymine on the opposite strand, and each guanine is connected by three hydrogen to a cytosine on the opposite strand. In each DNA molecule, the sequence of the bases on one strand is, therefore, complementary to the sequence of bases on the opposite strand.

DNA Base Ratios in Bacterial Taxonomy

The study of bacterial DNA is, at present, inapplicable to routine diagnostic work; however, the techniques of molecular biology do assist identification of isolates by refining bacterial taxonomy. In the future, techniques may become simplified enough to be used by the diagnostician (Hill, 1968).

Spirin et al. (1957) and Lee et al. (1956) were the first to recognize the importance of DNA base analyses as a taxonomic aid.

Chargaff proposed the basic rules underlying the concept of base ratios and taxonomic relatedness. In 1950, he asserted these rules: (1) The base composition is characteristic of the organism. (2) Different cells of the same tissue have approximately the same composition. (3) Base composition varies among organisms giving the dysymmetry ratio of $\frac{\text{adenine(A) + thymine(T)}}{\text{guanine(G) + cytosine(C)}}$ (4) Closely related organisms have the same base ratios. (5) a. The amount of A is equivalent to the amount of T. b. The amount of G is equivalent to the amount of C. c. The amount of A+T is equivalent to the amount of G+C. d. The amount of A+C is equivalent to the amount of G+T. e. C = cytosine and all substituted cytosines. f. T = uracil and all substituted uracils.

I. DNA Extraction

The isolation of native DNA is as much a part of the problem as is the actual determination of base composition. Fleming (1922) and Thompson (1949) were among the first workers to recognize the bacteriolytic effect of lysozyme, an enzyme present in bodily secretions, which attacks linkages between molecules of N-acetyl glucosamine and N-acetyl muramic acid. Lysozyme was employed as a method of DNA extraction, along with other chemical methods, by such workers as Vendreley (1956), Sarfert and Venner (1965), Poverennyi and Aleinikova (1964), Massie and Zimm (1966), and Oxenburgh and Snoswell (1965).

A combination of mechanical and enzymatic methods has been employed by a number of workers. Clausen, in 1961, described a method of isolating DNA from lyophilized cells by grinding with some abrasive agent. The lyophilization makes the cell wall more brittle and susceptible to breakage. However, Bradley (1968) indicated that lyophilization produces artifacts which affect DNA thermal denaturation studies, but not buoyant density determinations.

A very significant contribution by Marmur in 1961 was a generalized procedure for isolation of DNA. His method is a sequence of steps involving disruption of cells by mechanical means and/or enzymatic degradation, removal of cell debris and protein by denaturation, removal of RNA by the action of ribonuclease and selective precipitation of DNA with isopropanol. This procedure has been the most widely applied in base ratio studies and was used with modifications in my research.

Pfau and McCrea (1962) introduced a new method using pronase, a broad spectrum protease from Streptomyces griseus (Hiramatsu and Ouchi, 1963), for certain cells that are hard to lyse.

Smith (1963) employed numerous methods of cell disruption in an attempt to release DNA of several species of Actinomyces. A series of trials involving lysozyme, antibiotics, detergent, bacterial enzymes, a freeze press, sonic oscillator, and Servall Omnimixer was unsuccessful in yielding native DNA for subsequent base analysis.

Zimmer and Venner (1963) in the third of a series of studies on nucleic acids reported on the isolation of nucleic acids from Streptomyces using phenol, lysozyme, and a modification of the Marmur method. From 2-3 grams of the raw product, 0.5 to 1.0 gram of nucleic acid was obtained.

In 1963, Penzikova and Mikhailova showed that the Streptomyces as members of the Actinomycetales are susceptible to lysozyme, in contrast to the resistance of the Actinomyces.

Villaneuva (1966), in speaking of protoplasts of fungi, expressed the basic concepts that must be considered in lysing any cell to extract its DNA. The components of the cell wall have a great deal to do with its susceptibility to breakage. He states that the components of the cell wall owe their insolubility either to a polymeric of fatty nature. Also, a layering effect resists lysis. He states further that specific enzymes can be used to attack certain components of the wall. Physiological age and cultural conditions affect the fragility or rigidity of the cell wall. It was found that use of young cultures and certain substituted substrates such as melibiose make the walls more sensitive to lysis.

II. Determination of Base Ratios

Base ratios may be determined by (1) chemical analysis (2) spectral analysis (3) buoyant density and (4) denaturation temperature. The chemical method, largely superseded by the other physico-chemical methods, was employed by such workers as Marshak and Vogel (1951), Cohn (1955), Loring (1955), Holdgatre and Goodwin (1964), Wang and Hashagen (1964), Guschlbauer et al. (1965), Randerath (1965), MacGee (1966), Huang and Rosenberg (1966).

Spectral absorbancies were employed by De Ley (1967) for quick approximation of DNA base composition. He states that approximate base compositions of pure DNA can be quickly estimated from the absorbancy ratio of $E_{260\ m\mu} / E_{280\ m\mu}$ in 0.1 N acetic acid according to the empirical relat-

ionship %GC = 168.6 - 87.4 ($E_{260\ m\mu}$ / $E_{280\ m\mu}$), valid in the range of 40-70% GC. The method is accurate within 3% GC. It can be used when a quick, rough estimation of DNA base composition is required, e.g., to check correct taxonomic position of new isolates or to approximate T_m or buoyant density of an unknown DNA. The method cannot be recommended for distinguishing between two genera with closely-related GC values, or for finer distinction within one genus.

Other workers which have utilized spectral analysis are Hirschman and Felsenfeld (1966) and Fritzsche (1967) who used the infrared spectra in differentiation of base ratios.

Buoyant densities in relation to base ratios was first described by Meselson, Stahl, and Vinograd (1957) who utilized the fact that the percentage of guanine + cytosine is linearly related to the corresponding buoyant density of the bases. Schildkraut, Marmur, and Doty (1962) then outlined a specific method utilizing this buoyant density theory. The advantages of such a procedure are that only 1 µg of DNA in a crude state is needed and certain unusual bases are detected. The disadvantages are that only total GC content is given and certain substances such as glucose may give erroneously high values for the GC content. Cheng and Sueoka (1963) described the effect of the heterogeneity of DNA density on the base composition, only emphasizing the fact that the GC values obtained in this manner are only averages.

Michelson (1958) first reported the hyperchromic characteristics of nucleic acids as related to thermal denaturation. Heating of the double-strand DNA causes a separation of the two complementary strands of native DNA, which causes a sudden increase in absorbance in a narrow temperature range (hyper-

chromic shift). The increase in absorbancy is due to the separation of the two strands resulting in the liberation of the nitrogenous bases from the hydrogen bonding which had a suppressing effect on their absorbancy. This is carried out at 260 mμ, the peak absorbance wavelength for DNA, due to the nitrogenous bases.

Marmur and Doty (1962) then employed Michelson's theory on the hyperchromicity of nucleic acids and published a method for determination of DNA base composition from its thermal denaturation temperature (T_m). This method requires 10-50 μg of DNA, which must be highly polymerized. Freund and Bernardi (1963) restudied the hyperchromic properties of DNA and established that a change in intrinsic viscosity parallels the change in absorbance at 260 mμ except for the "sub-melting" temperature range. Zimmer and Venner (1963) in a series of studies on nucleic acids, described the dependence of DNA thermal denaturation on pH. The GC dependence on the T_m becomes obvious at pH 7.0 and has been found to be practically zero at pH 3.0. The more strongly reduced T_m values at pH 3.0 of DNA's rich in GC compared with those DNA's rich in AT are due to relatively greater labilization of GC base pair interactions, caused by higher protonation of the GC paris. Takashima and Arnolds (1965) made kinetic studies on thermal denaturation of DNA with a high-frequency heating method in order to elucidate the T_m determination process. They discovered that the rate of heat denaturation of bacterial DNA is rather slow, requiring more than 3 seconds. The rate is dependent on the GC content and is faster when the GC content is lower; rates are also faster below pH 5.0.

III. Representative Base Ratios

Actual values for DNA bases were determined long before their genuine value in taxonomy was recognized. Almost every genus is represented in the literature on GC content reported. Johnson and Brown (1922) isolated nucleic acid of the thymus type (DNA) from tubercle bacilli and determined the AG content. Vischer et al. (1949) and Chargaff et al. (1949) determined base compositions of spleen, thymus, salmon sperm, and yeast DNA using paper chromatography. Smith and Wyatt (1951) were among the first to realize that the composition of DNA is characteristic of the species source.

Spirin et al. (1957) studied species specificity with respect to nucleic acids in bacteria and found that the only satisfactory method for study of qualitative differences in nucleic acids is determining quantitative proportions of their nitrogen bases. They further stated that nucleotide composition of DNA in bacteria shows extensive variation from one species to another, displaying a whole range of diversity in possible types, from the most extreme AT type to the highest of those GC types known, and all the intermediate relationships.

Belozersky and Spirin (1960) recognized not only the variation of nucleic acid content, but also the stability of base ratios with such factors as growth phase, conditions of cultivation, physiological and functional state of the cells, as well as on the strain itself.

Sueoka (1961) compiled old and new data to illustrate variation and heterogeneity of base composition of DNA. Among bacteria, the mean GC content of DNA varies approximately 25-75% and this extends over the range of the mean GC content of DNA of higher organisms. Distribution of GC content of DNA molecules of an organism is generally unimodal and the range is rel-

atively narrow. If the mean GC content of DNA of two species is different by 10% in bacteria, there are few DNA molecules of the same GC content common to the two species. Absence of natural DNA outside this range may suggest either that the genetic information cannot be stored by adenine and thymine or guanine and cytosine alone. Bacterial DNA has a narrower distribution than that of higher organisms. The range of compositional heterogeneity of DNA molecules in terms of GC content has been observed in bacterial species to be less than 6% (expressed as twice the standard deviation) about the mean GC content.

On the genetic basis of variation and heterogeneity of DNA base composition, Sueoka (1962) stated a unitary theory based on genetic and evolutionary considerations and which attempts to account for the main characteristics for distribution of DNA base composition in nature. Conversion of base pairs causes a change of GC content and therefore, the mutation rate of genes is related to base composition. Only conversions which survive and establish themselves in the following generation contribute to the variation of GC content. The main assumption in the present theory is that each mutagenic factor acts rather uniformly on base pairs of DNA without regard to their location in the chromosome or in the DNA molecules. There are two lines of thought concerning phylogenetics: (1) Similarity in DNA base composition among closely related organisms comes from their common origin and from the stable nature of the base composition. (2) Organisms with a similar internal environment have a similar weight per volume ratio, thus making their base compositions alike.

Marmur (1963) reported on the role of informational molecules such as DNA in genetic transfer, transformation, and transduction. Colwell and

Mandel (1964) employed a numerical taxonomy in estimating phylogenetic positions of organisms whose base ratios were determined from thermal denaturation curves. Hill (1966) recently reviewed base compositions of several genera and published an index of various bacterial species. DNA homologies as differentiated from DNA base analyses in taxonomic importance is indicated by De Ley et al. (1966) in the need for doing homologies, as well as base analyses, to present the complete picture of genetic relatedness or nonrelatedness.

In a study of DNA of Actinomycetales, Bouisset et al. (1965) compared A+T/G+C ratios with bacteriological properties of the corynebacteria. These studies indicated a relationship between the ratios obtained and the enzymatic capacity of different groups of species. This might support Sueoka's theory, if the genetic code is universal, that there may be some correlation between GC content of DNA and amino acid content of protein.

In a series of studies on nucleic acids, Zimmer and Venner (1964) reported the thermal denaturation of DNA from Streptomyces sp. They studied the effect of proteins and polysaccharides on the helix-transfer of the streptomycete DNA, and found that the hyperchromic effect is in all cases virtually unchanged. Especially in cases of streptomycete DNA, it is not always easy, in spite of careful purification, to obtain satisfactory material.

Frontali et al. (1965) made a taxonomical study of Streptomyces using DNA base ratios determined by thermal denaturation and buoyant density. Using Marmur's extraction procedure, in addition to lysis with 2-mercapto-ethanol, DNA was obtained for base analysis. However, they did find a difference between GC values obtained from buoyant density and those from T_m.

Craveri et al. (1965) made a study of base compositions of thermophilic Actinomyces. Using procedures similar to those of Frontali, he

obtained values for two species with unusually low GC content. He attributes this to an unusual base in the DNA molecules and continues to surmise that organisms with different DNA base ratios may have, nonetheless, a common origin.

Lawrence and Clark (1966) characterized the DNA from Streptomyces scabies using lyophilized cells for chemical and thermal denaturation studies. Their statement that those results based on T_m studies were the only ones in the stictest sense comparable cannot now be entirely relied upon, due to the recent indication of the effects of lyophilization.

A very recent report of DNA for Streptomyces and Nocardia is that of Tewfik and Bradley (1967) where relationships among selected Streptomyces, Nocardia, and Mycobacterium were determined, based on the GC content of their DNA and on the ability of denatured DNA to anneal with single-stranded reference DNA. It was found that Streptomyces constitute a homogeneous group whose DNA has a 69-73 mole %GC; Nocardia and Mycobacterium had 62-69 %GC. It was also noted that buoyant density of DNA from S. venezuelae spores was lower than that from the mycelia.

Yamaguchi (1967) made DNA base ratio and homology studies of various morphologically distinct actinomycetes in order to find out any genotypic difference that may exist between their DNA's, for a more rational approach to the taxonomy and phylogeny of the actinomycetes. His results, obtained by paper chromatography indicated a range of 67-74 %GC for Streptomyces and 70-74 %GC for Nocardia, but the difference was gradual and failed to delineate any organisms. His results, as stated by himself, indicate that, in spite of various morphological differentiations both in aerial and vegetative hyphae, most actinomycetes have similar and very high GC content among

microorganisms, suggesting a culmination of evolutionary diversification in procaryotic organisms.

Gasser and Sebald (1966) reported the nucleic acid base composition of the genus Lactobacillus. Gasser and Mandel later made a follow-up study (1968) on this genus, in which DNA of 45 strains of Lactobacillus and 5 strains of Bifidobacterium which had been analyzed for base composition by chromatographic means were examined at equilibrium in cesium chloride density gradient. Regression analysis showed that there had been systematic errors involved in the estimation of GC content by the chemical method, and that the relation between buoyant density and base composition is indeed linear and best fitted by the equation %GC = 10.39 (buoyant density - 1.662), which compares well in slope with the equation of Schildkraut, Marmur, and Doty (1962).

The purpose of this investigation is to obtain base ratio values of the Actinomyces spp. and related diphtheroids, in hopes of ascertaining their taxonomic significance.

MATERIALS AND METHODS

Organisms

The organisms used throughout this study are listed in Table 1. All known species are American Type Culture strains (ATCC) unless otherwise indicated as originating from the Communicable Disease Center (CDC). The diphtheroids were selected from those isolated by Biever (1967) from cattle and swine obtained from Armour & Co. in Huron, S. D.

Table 1. Sources of Reference Organisms

Name	Source number	Source
Actinomyces bovis	ATCC 13683	ATCC*
Actinomyces israelii	ATCC 12102	ATCC
Actinomyces naeslundii	ATCC 12104	ATCC
Actinomyces eriksonii	ATCC 15423	ATCC
Actinomyces odontolyticus	ATCC 17929	ATCC
Actinomyces parabifidus	ATCC 17930	ATCC
Actinomyces proprionicus	ATCC 14157	ATCC
Actinomyces discofoliatus	W-859	NCDC**
Odontomyces viscosus	ATCC 15987	ATCC
Ramibacterium pleuriticum	ATCC 19301	ATCC
Rothia dentocariosus	ATCC 17931	ATCC
Corynebacterium pyogenes	W-972	NCDC
Corynebacterium haemolyticum	W-969	NCDC
Corynebacterium acnes	X-424	NCDC

*American Type Culture Collection, Washington, D. C.

**National Communicable Disease Center, Atlanta, Georgia

Table 1. (continued)

Isolate number	Source
230	Swine Udder
327	Swine Udder
333	Swine Udder
179	Swine Jowl
24	Swine Jowl
144	Swine Liver
130	Bovine Liver
168	Swine Udder
221	Bovine Liver
288	Bovine Liver
217	Bovine Liver
279	Swine Jowl
303	Swine Udder
166	Swine Udder

Preliminary Cell Preparation

I. Preparation of Inoculum

A. Frozen cultures (previously preserved at -20 C in Trypticase Soy Tryptose (TST) + Fluid thioglycollate + 0.35% agar) were transferred to tubes of TST + Fluid thioglycollate and incubated 3-5 days at 37 C.

B. Two more consecutive transfers were made in this way, the final to two tubes per organism to provide inoculum for mass cultures. To insure purity, Gram stains were made using Hucker's modification.

Trypticase Soy Tryptose + Fluid thioglycollate

Fluid thioglycollate(without dextrose)	24 g
Dextrose	5 g
Trypticase Soy Broth	1.5 g
Tryptose Broth	1.25 g
Distilled water	1000 ml

II. Mass Culture of Organisms

A. Fresh inoculum (2.0 ml) was used to inoculate each of ten dilution bottles containing 100 ml of the media (TST + NIH thioglycollate broth) per organism. The caps were screwed tightly immediately to maintain anaerobiosis and the bottles were incubated at 37 C according to the following schedule:

Culture	Time (in days)	Conditions
A. bovis	5	Anaerobically
A. israelii	5	Anaerobically
A. naeslundii	5	Anaerobically
A. eriksonii	5	Anaerobically
A. odontolyticus	5	Anaerobically
A. parabifidus	5	Anaerobically
A. proprionicus	5	Anaerobically
A. discofoliatus	2	Anaerobically
O. viscosus	2	Anaerobically
R. pleuriticum	5	Anaerobically
R. dentocariosus	5	Anaerobically or aerobically
C. haemolyticum	2	Aerobically
C. pyogenes	2	Aerobically
C. acnes	5	Anaerobically
All isolates of Table 1.	5	Anaerobically

Trypticase Soy Tryptose + NIH thioglycollate broth

NIH thioglycollate broth (without agar), Difco	28.5 g
Trypticase Soy Broth	1.5 g
Tryptose Broth	1.25 g
Distilled water	1000 ml

B. Gram stains were made of each bottle to insure purity.

C. At approximately the logarithmic phase of growth, the cells were harvested by centrifugation at 3500 rpm for 30 minutes on the International Refrigerated Centrifuge (International head no. 822). The

cells were washed twice in 50 ml portions of saline-EDTA, pH 8.0.

III. Drying of Organisms

A. Each five grams of cells, wet weight, were washed in 100 ml of 95% ethanol, centrifuged slowly, and the supernatant liquid poured off.

B. The cell pellet was then washed in 50 ml of anhydrous acetone + 50 ml of 95% ethanol, centrifuged slowly, and the supernatant liquid poured off.

C. Finally, the cell pellet was washed in 100 ml of anhydrous acetone, centrifuged slowly, and the supernatant liquid poured off. The cells were left overnight to air dry, and subsequently stored at -20 C for further use.

Nucleic Acid Extraction

I. Mechanical Rupture

A. One-half gram of dried cells + 1 gram 20 μ acid-washed glass powder (Minnesota Mining and Manufacturing, St. Paul, Minnesota) + a 2 inch cube of pulverized dry ice were ground vigorously for 5-10 minutes in a pre-cooled mortar and pestle. Dry ice was added at intervals to keep the mixture cold and dry.

II. Extraction

A. Generally, the Marmur method was used, except that lysozyme was substituted with pronase.

B. One-half gram of dried mycelial material was added and ruptured as described above.

C. Ten ml of saline-EDTA and enough pronase to make a 500 μg final con-

centration were added to the mixture and then incubated one hour at 42 C with occasional agitation.

D. Two ml of 25% sodium lauryl sulfate was added to the pronase + EDTA + cell mixture and incubated 15 minutes at 60 C with occasional shaking.

E. After cooling in cold tap water, 7 ml of 5M sodium perchlorate or enough to give at least a 2M sodium perchlorate concentration in the total solution was added and the mixture shaken for 5 minutes. The high salt concentration dissociates DNA from protein.

F. Thirty ml of Sevag's mixture was added and shaken 15 minutes on a wrist action shaker.

G. The three layers were separated by centrifugation at 8-10,000 X g for 5-10 minutes on the Servall SS-34. The top layer (aqueous layer) contains the nucleic acids, the middle contains denatured protein, and the bottom contains the chloroform and glass beads.

H. The nucleic acid aqueous layer was carefully collected using a 10-ml volumetric pipette (a propipette works well here), being cautious not to take up the protein layer. Also, a wide bore pipette eliminates any shear forces that may denature DNA.

I. The nucleic acid fraction was then transferred to a 125-ml glass-stoppered Erlenmeyer flask and gently overlaid, with as little mixing as possible, 25 ml (2 vol) of 95% ethanol.

J. The nucleic acid strands were collected by spooling the precipitate on a small-diameter glass-stirring rod. The collection was completed by swirling the layers together and precipitating again.

K. The collected nucleic acids were dissolved in 4.5 ml of 1 X SSC (standard saline citrate). The nucleic acids must be completely dissolved

and rehydrated before continuing the purification, which takes over an hour. Therefore, the collected nucleic acids can be stored at 5 C overnight to dissolve.

L. To further the purification, the rehydrated nucleic acids were transferred to a glass-stoppered flask, and enough ribonuclease was added to give a total concentration of 50 µg/ml. The mixture was incubated at 37 C for 30 minutes. If the solutions were cold or cool, enough time was allowed to insure 30 minutes at 37 C. This will remove any RNA in the fraction.

M. Five ml of Sevag's mixture was added and shaken for 30 minutes on a wrist action shaker to deproteinize.

N. Steps G, H, I, and J were repeated.

O. The crude DNA was dissolved in 4.5 ml of 0.1 X SSC in a clean screw-cap test tube and allowed to rehydrate; thus, it may be left at 5 C overnight.

P. When the DNA was in solution, it was transferred to a large screw-cap tube and 1.0 ml of 3M sodium acetate-EDTA was added and mixed gently.

Q. Three to five ml of isopropanol was added dropwise while the solution was stirred rapidly, until the DNA precipitated. This leaves the RNA fragments in solution.

R. The DNA was harvested by spooling on a glass rod. If the DNA preparation was broken up (due to excessive grinding), it was centrifuged at low speed (1000 rpm) for 10 minutes.

S. The DNA was dissolved in 4.5 ml of 1 X SSC in a small, clean screw-cap test tube and stored at 5 C over several drops of chloroform. Stability is good for at least six months.

Reagents for DNA Isolation

Saline-EDTA, pH 8.0 — 0.15 M NaCl + 0.1 M ethylenediaminetetra acetate (EDTA)

NaCl	8.7 g
EDTA	29.23 g
Distilled water	1000 ml

Nine hundred ml of water was added, and then saturated NaOH was added until most of the material dissolved. The pH was then carefully adjusted to 8.0 using 1.0 N NaOH. The volume was then brought to 1000 ml with distilled water. The EDTA and/or high pH inhibit DNAase activity.

Sodium lauryl sulfate, 25% (SLS)

SLS	25 g
Distilled water	100 ml

The detergent did not dissolve completely; only the supernatant liquid was used. The anionic detergent ($NaC_{12}H_{26}SO_4$) lyses most nonmetabolizing cells, inhibits enzyme action, and denatures some proteins.

Pronase, 10 mg/ml

Pronase (B grade, Calbiochem 53702)	0.2 g
Distilled water	20 ml

The solution was dispensed in tubes, 5 ml per tube, and stored at -20 C until used. Pronase is a broad-spectrum protease extracted from *Streptomyces griseus* and lyses many resistant cells.

Sodium perchlorate, 5M

$NaClO_4$ 306.25 g

Distilled water 500 ml

The volume was brought to 500 ml with distilled water. The solution cooled considerably because the heat of solution is endothermic. In order to speed dissolution, the flask may be placed in a beaker of hot water. The high salt concentration provided by the perchlorate helps dissociate protein from nucleic acid.

Sevag's Mixture

Chloroform 24 parts

Iso-amyl alcohol 1 part

This is used to deproteinize, according to the method of Sevag, Lackmann and Smolens. The chloroform causes surface denaturation of proteins. The iso-amyl alcohol reduces foaming, aids the separation, and maintains the stability of the layers of the centrifuged, deproteinized solution.

Ethyl alcohol, 95%

This is used to precipitate nucleic acids following deproteinization. Denatured alcohol may also be used.

Saline-citrate, standard 0.15 M NaCl + 0.015 M Na citrate, pH 7.0

10 X SSC:

	NaCl	43.84 g
	$Na_3C_6H_5O_7 \cdot 2H_2O$	22.06 g
	Distilled water	400 ml

Usually a 10 X SSC is prepared (1.5 M NaCl + 0.15 M Na citrate, pH 7.0) and diluted to obtain 1, ½, and 1/10 X SSC. After dissolving all materials, the pH was adjusted to 7.0 with 1.0 N NaOH. The volume was then brought to 500 ml with distilled water. DNA dissolves more readily in dilute salt solutions but should never be dissolved in pure water. The concentrated solution (10 X SSC) is used to bring the dilute saline-citrate solute (1/10 X SSC), in which the nucleic acid is dissolved, up to standard saline-citrate concentration (1 X SSC). The volume added need only be approximate until the final product is obtained. Standard saline-citrate maintains ionic strength of dissolved DNA and chelates divalent ions, as well as inhibiting nucleases.

Ribonuclease, 0.2% in 0.15 M NaCl, pH 5.0

RNAase (crystalline, Armour)	40 mg
0.15 M NaCl	20 ml

The solution was heated at 80 C for 10 minutes to inactivate any contaminating DNAase. The RNAase digests the RNA and facilitates its separation from DNA.

Acetate-EDTA, 3.0 M sodium acetate + 0.001 M EDTA, pH 7.5

NaC_2H_3O	40.83 g
EDTA	0.029 g
Distilled water	90 ml

The pH was adjusted to 7.5 with 1.0 N NaOH and the volume brought to 100 ml. This provides the proper ionic environment in the isopropanol step for the separation of DNA from RNA or its digestion products.

Isopropanol

This is used to precipitate DNA selectively; RNA remains in solution. In some cases it will selectively precipitate and separate DNA from polysaccharides.

Equipment for DNA Isolation

Centrifuge, Servall SS-34 operating at 8000 rpm

Glass-stoppered flasks, for deproteinization

Shaker, reciprocal for deproteinization, at several hundred strokes/minute

Volumetric pipette, 10 to 15 ml, fitted with propipette

Water baths, 37 C, 42 C, and 60 C

Physical Measurements

I. Determination of Thermal Denaturation Midpoint

A. DNA stock solution (in 1 X SSC) was diluted 1:10 with distilled water to bring it to 0.1 X SSC.

B. The $O.D._{260\ m\mu}$ and $O.D._{280\ m\mu}$ was determined of this new stock solution using the Zeiss UV spectrophotometer. The $O.D._{260\ m\mu}/O.D._{280\ m\mu}$ should be at least 1.8, indicating a tolerable amount of protein in relation to nucleic acid. If this ratio is too low, the DNA should be reprecipitated with isopropanol.

C. The new stock solution was properly diluted with 0.1 X SSC to obtain an $O.D._{260\ m\mu}$ of about 0.48, which corresponds to a total content of 20 µg.

D. The four quartz cuvettes to be placed in the Gilford Multiple Sample Absorbance Recorder were filled as follow:

Cuvette no.	Contents
1 - Blank	3 ml 0.1 SSC (O.D. 0.00)
2 - DNA	3 ml sample (O.D. 0.48)
3 - DNA	3 ml sample (O.D. 0.48)
4 - DNA	3 ml sample (O.D. 0.48)

The cuvettes were then stoppered and placed in the special cuvette holder.

E. The Gilford Absorbance Recorder (Model 2000) was calibrated with the blank set at an absorbance of 0.00. The heating element was then activated and a slow, constant increase (1^{o} per minute) of temperature was begun.

F. Duplicate runs were made for each sample, with Corynebacterium pyogenes DNA (%GC = 48.5) as a reference DNA.

G. The midpoint temperature corresponding to one-half of the absorbance increase was extrapolated from the tracings such as shown in Figure 1.

H. Duplicate T_m values were averaged to obtain the T_m to be used in the calculation of GC content.

Determination of Base Composition

I. Experimental Graphic Method

As shown in Figure 2, two samples of known GC content were used to determine the slope of a line from which could be extrapolated the GC content of unknown samples from their T_m. The two reference DNA's used in this study were from Corynebacterium pyogenes, with a GC content of 48.5 % as quoted by Hill (1966) as determined chemically, and from Actinomyces bovis with a GC content of 64.0 % as determined by the author using a value arrived at by

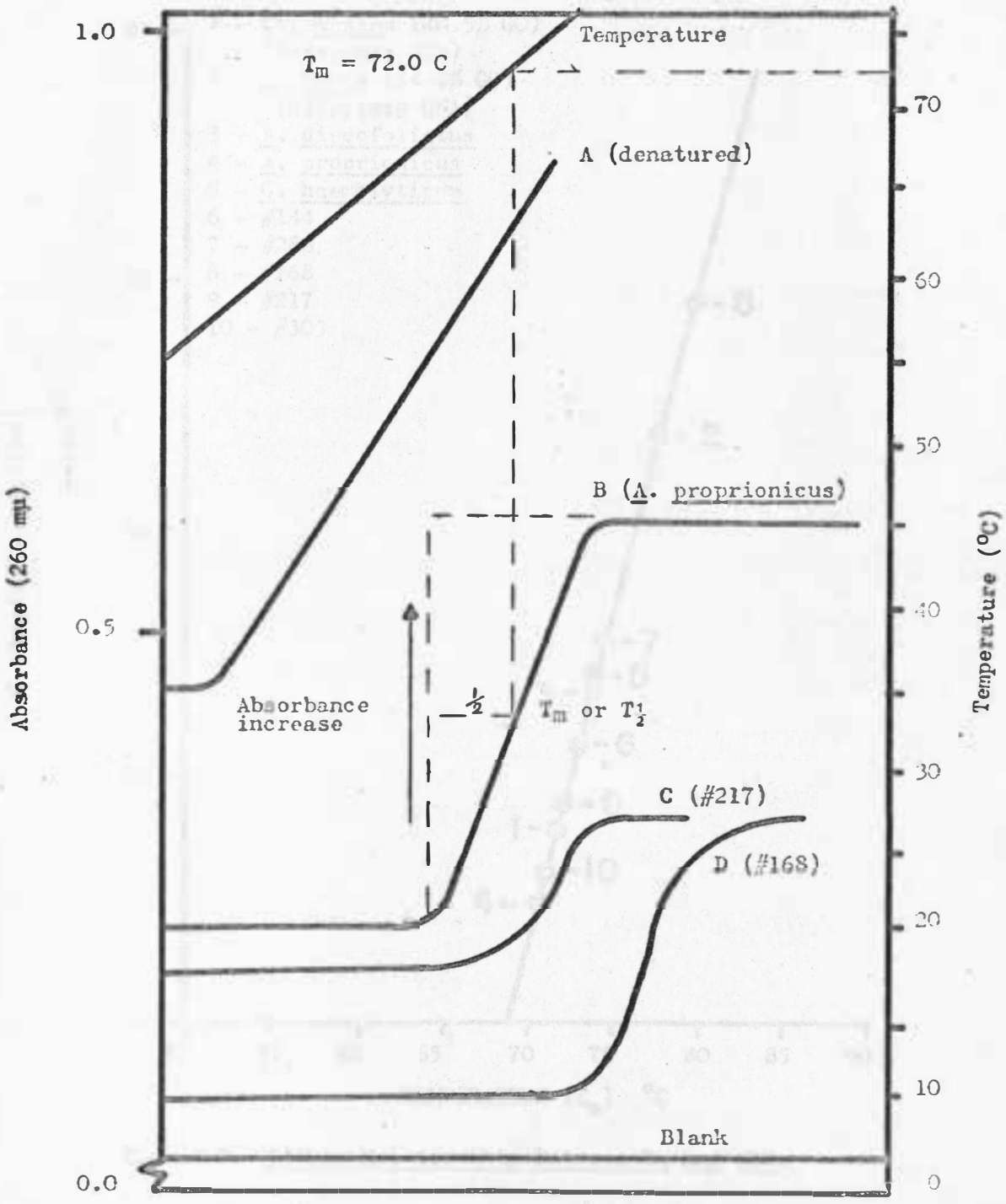

Figure 1. Representative T_m Curves of DNA from Actinomyces proprionicus and Two Related Diphtheroids.

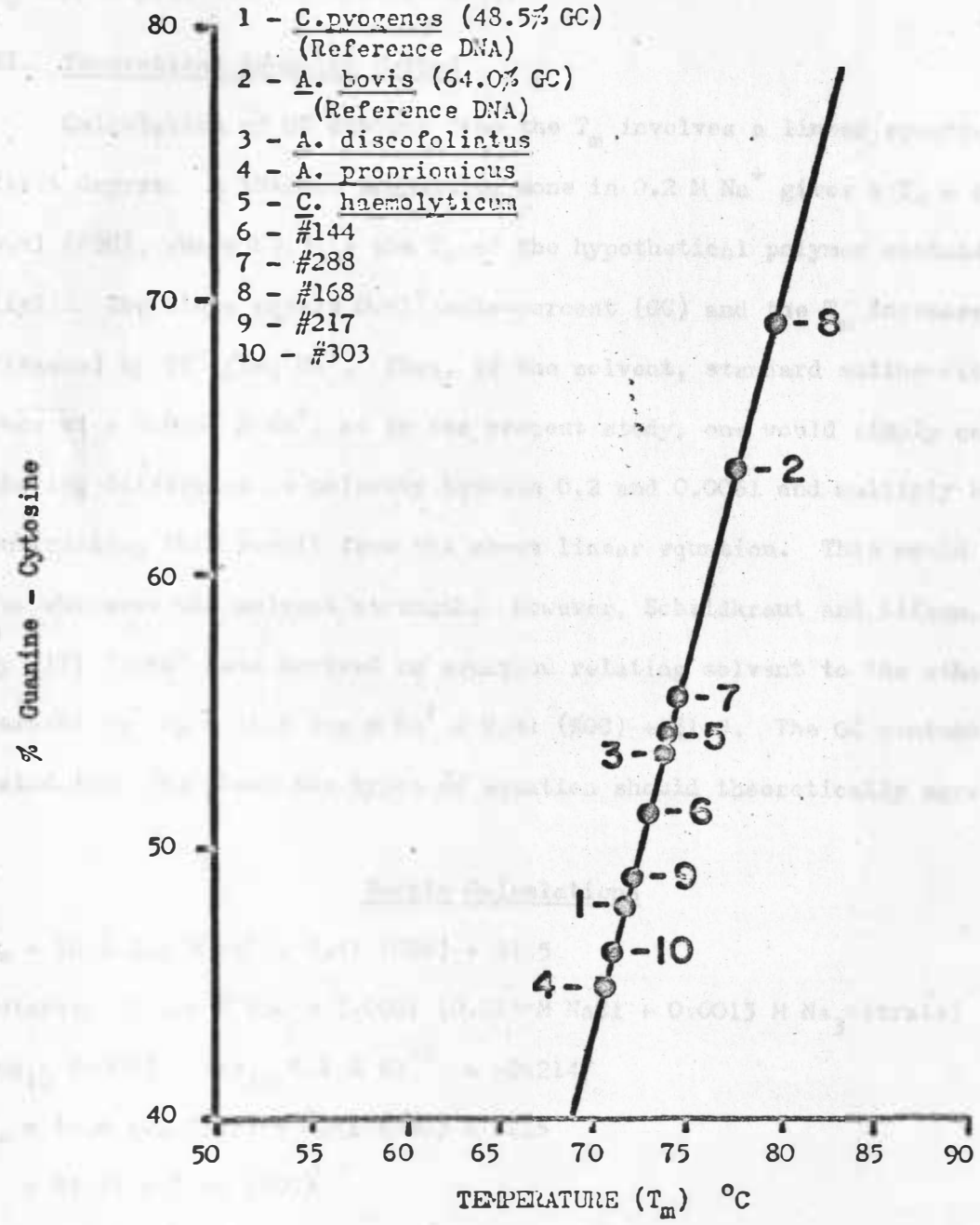

Figure 2. Linear Relationship Between T_m and %GC.

T_m and buoyant density measurements.

II. Theoretical Equation Method

Calculation of GC content from the T_m involves a linear equation of the first degree. A thermal transition done in 0.2 M Na^+ gives a T_m = 69.3 + 0.41 (%GC), where 69.3 is the T_m of the hypothetical polymer containing only (A+T). The slope equals 0.41°/mole-percent (GC) and the T_m increases (or decreases) by 18° /log Na^+. Thus, if the solvent, standard saline-citrate were at a 0.0061 M Na^+, as in the present study, one would simply calculate the log difference in molarity between 0.2 and 0.0061 and multiply by 18, subtracting this result from the above linear equation. This would be done for whatever the solvent strength. However, Schildkraut and Lifson, as cited by Hill (1968) have derived an equation relating solvent to the other two variables: T_m = 16.6 log M Na^+ + 0.41 (%GC) + 81.5. The GC content calculated from the above two types of equation should theoretically agree.

Sample Calculations

$T_m = 16.6 \log M\ Na^+ + 0.41\ (\%GC) + 81.5$

Molarity of 0.1 X SSC = 0.0061 (0.015 M NaCl + 0.0015 M Na_3citrate)

$\log_{10} 0.0061 = \log_{10} 6.1 \times 10^{-3} = -2.2147$

$T_m = 16.6\ (-2.2147) + 0.41\ (\%GC) + 81.5$

$= 44.74 + 0.41\ (\%GC)$

$$\%GC = \frac{T_m - 44.74}{0.41}$$

For Actinomyces discofoliatus (T_m = 74.5 C), $\%GC = \frac{74.5 - 44.74}{0.41} = 72.6\ \%$

RESULTS AND DISCUSSION

Lysis and Subsequent DNA Extraction

Before the DNA of any specific organism can be successfully characterized, it must be isolated in a relatively pure, native form. With some of the organisms in the Actinomyces study, this was not possible. The modified Marmur procedure utilized as a basis for disrupting the Actinomyces is, in general, effective when large amounts of cell material are available for lysis. Young, logarithmically-grown cells in an agar-free medium are most susceptible to lysis under minimum conditions, which do not denature DNA.

As a member of the Actinomycetales, the Actinomyces follow the trend of the Streptomyces, Nocardia, and Mycobacteria in the refractory nature of their cell walls. Thus, mechanical grinding with glass powder promised the most efficient way, however crude, of rendering the cell walls vulnerable to subsequent enzyme action. Grinding with a glass bead of mesh size comparable to that of the organism is thought to physically uncover enough active sites on the cell wall to permit the enzyme(s) to breakdown the cell wall components.

Formerly it was thought that lyopholization was suitable in converting the cell wall to a more brittle state more apt to "crack" during the grinding process. However, lyopholization produces artifacts that cause a shift to a higher T_m during thermal denaturation of the DNA, as reported by Bradley (1968) who suggested a pre-extraction with ethanol and acetone as a substitute. Supposedly the dehydration and lipid extraction expose the cell wall to mechanical and enzymic attack in a process comparable to that of lyopholization, but without any effect on T_m determinations.

In reference to Table 2, organisms cultivated and harvested under identical conditions and extracted with the same modified method of Marmur, involv-

ing use of pronase, show a variation in susceptibility to lysis and in DNA yield. For those resistant organisms, numerous attempts in a trial and error fashion to substitute lysozyme, increasing incubation times and temperatures, were carried out to obtain some degree of lysis. In no case was lysozyme effective without pronase. Also, in some cases when lysis was effected, the DNA was degraded or denatured. This may have occurred during the mechanical grinding or as a result of a too high incubation temperature. The mild shear forces of shaking during deproteinization may also have destroyed some DNA.

The cell wall analyses carried out by Scheetz (1969) would be of considerable aid in selecting further enzymes or agents which could effectively attack the cell wall without denaturation of the DNA. In my study, I attempted to find some consistent component of the cell wall which was present or absent in the resistant cells as compared to the lysable cells. With the available data on cell walls, this was not possible; perhaps the several unidentified components hold the answer.

Determination of Thermal Denaturation Midpoint of DNA (T_m)

Overall base composition of a DNA can be estimated from the hyperchromic shift at 260 mμ when DNA solutions are heated slowly in appropriate buffers (Marmur and Doty, 1959). The midpoint of the hyperchromic shift, abbreviated T_m, of slowly-heated DNA, in appropriate salt solutions, increases as the mole-% of guanine-cytosine of DNA increases. The shape of the thermal denaturation curve also reflects the heterogeneity of the DNA (Figure 1).

The relative T_m values for each of the curves, B, C, and D can be expressed as $B < C < D$. Heterogeneity can be inferred from the slope of the absorbance increase. The steeper slope of curve C as compared to the broader

Table 2. Degree of Cell Lysis and DNA Extraction of Actinomyces and Actinomyces-like Organisms

Organism	Lysis*	DNA extracted in native form
Actinomyces odontolyticus	+	Yes
A. bovis	++	Yes
A. discofoliatus	+++	Yes
A. proprionicus	+++	Yes
A. israelii	-	No
A. parabifidus	-	No
A. eriksonii	+	No
A. naeslundii	-	No
Corynebacterium haemolyticum	+++	Yes
C. pyogenes	+++	Yes
C. acnes	-	No
Odontomyces viscosus	-	No
Rothia dentocariosus	-	No
Ramibacterium pleuriticum	-	No
Diphtheroid Isolates		
230	-	No
327	+	No
333	-	No
179	+	Yes
24	-	No
130	++	No
144	+++	Yes
221	-	No
288	+++	Yes
168	+++	Yes
217	+	Yes
279	+	No
303	+++	Yes
166	+++	Yes

*Lysis was judged on apprent increase of viscosity, qualitatively only

(-) = No lysis
(+) = poor
(++) = fair
(+++) = good-excellent

slope of curve B indicates that organism C has a rather homogeneous DNA, whereas organism B is more heterogeneous. Curve A represents the curve of a denatured sample of DNA, or, in other words, that of a single-stranded DNA. The slope is shallow, variable and ever-increasing which shows that individual helical segments melt independently of each other at temperatures that depend on their length and composition.

T_m values reported in Table 3 are averages of duplicate runs of DNA samples from each organism. Before analysis of the T_m values obtained in this study, it would be well to review the theory of the denaturation process as part of T_m determination. At the other extreme there is a completely disordered conformation: two separated, flexible random coils; at the other extreme we find a completely ordered double helical conformation. With DNA, the dominant factor in controlling the helix-to-random coil transition appears to be the additional stabilization energy arising from the mutual interactions of adjacent "stacked" bases. The main contribution to helix stability, then, is the force that has been variously referred to as hydrophobic or nonpolar force, as well as the hydrogen bonding between the complementary bases. It happens that the guanine-cytosine bond involves three hydrogen bonds, while the adenine-thymine involves only two; thus, the more stable helix (with a higher GC content) "melts" at a higher temperature.

The transition from the helical to random coil state can be represented by a simple sigmoid (Figure 1) defined by two parameters: the position of their midpoint and the steepness of the slope at this point. If the transition measured is a thermal one (the independent variable is temperature), then the transition becomes analogous to the melting of a uni-dimensional crystallite and the temperature at this transition midpoint ($T_{\frac{1}{2}}$) is equi-

Table 3. Base Composition of Actinomyces spp. and Actinomyces-like Organisms

Organisms	T_m	%GC	
	C	Graphic	Calculated
Actinomyces bovis	77.8	64.0	80.6
A. discofoliatus	74.5	53.1	72.6
A. proprionicus	72.0	45.0	66.5
Corynebacterium pyogenes	73.0	48.5	68.9
C. haemolyticum	74.8	55.0	73.3
Group I*			
144	74.0	51.8	71.4
Group III			
288	75.0	56.0	73.8
168	80.0	70.0	86.0
Group IV			
217	73.1	49.5	69.2
303	72.1	46.0	66.7
166**	72.1	46.0	66.7

*Biever's groupings

**Regrouped as result of base analyses

valent to a melting point (T_m). Doty and coworkers demonstrated that the T_m shows a direct functional dependence on the composition of the DNA, increasing with increasing GC content. No such dependence is found with respect to transition width. The extent of the hyperchromic shift is proportional to the change in helical content; a complete helix-coil transition causes an increase of about 40%. A plot of the $O.D._{260\ m\mu}$ as a function of temperature yields a sigmoid curve whose slope is steep for pure helices, in which the shift from helix to random coil occurs rapidly in the molecules.

Factors which influence T_m include ionic strength. Denaturation temperature is linearly related to the logarithm of the solvent ionic strength; the more dilute the solvent, the lower the T_m. Needless to say, presence of materials other than DNA which absorb at 260 mμ would interfere. A protein content of more than that indicated by a 2:1 ratio of the $O.D._{260m\mu}$ / $O.D._{280\ m\mu}$ from the UV readings would also alter readings. T_m is essentially unaltered by degrading the double-stranded molecules. Presence of divalent ions and polyamines, as well as the rate of heating, affect the T_m.

Advantages of T_m determination are clear. The result can be obtained easily on a very small amount of DNA, ordinarily 20-50 μg, although 15 μg suffice with special cuvettes. Three samples can be determined in one hour.

Limitations of the method are its insensitivity to other than the four normally-occuring bases and its restriction to a range of composition of 25-75 %GC; that is, below and above, there is no linearity between T_m and GC content.

Those organisms from which DNA was obtained in sufficient amounts for analysis are tabulated with T_m and %GC in Table 3. To evaluate reproducibility of T_m values, a number of determinations on portions of the same

sample and on different preparations from the same source should be carried out. In the case of the Actinomyces, different portions of the same samples were run in duplicate; however, portions of different DNA preparations were not carried out because of the difficulty in obtaining enough, if any, DNA for a minimum number of runs. Moreover, T_m values of several strains within a species must be determined, since genetic mutations in changing the bases, change the GC content.

Base Composition as Determined from Thermal Denaturation

A survey of the literature to the end of 1965 shows that DNA base compositions have already been reported for about 100 differently named species; thus, DNA base compositions are becoming widely used in taxonomy and it is already evident that there is a need to include such data in future species descriptions. This stage has been reached for all members of the Actinomycetales, except the Actinomyces, for which no base ratios have as yet been published. The confusion of the Actinomyces makes it especially important to obtain base ratios for this genus, as well as other related anaerobic diphtheroids. Methods of determining GC content are from (1) Chemical analysis (2) Denaturation temperature (3) Buoyant density and (4) Spectral analysis. Availability of apparatus within the allotted time necessitated the use of the denaturation method in obtaining GC content. Usually the thermal denaturation and buoyant density method are used in conjunction and have been found to agree with one another.

Table 3 summarizes the GC content of those diphtheroids whose DNA was successfully isolated in native form. The values are those obtained by the graphic and theoretical equation method. There is a significant difference

between the empirically-obtained values and those calculated from the theoretical equation. The graphically-obtained values are based on all the factors within this particular system, but the equation theorized for a more general application does not account for any possible variations inherent in the system. Thus, the GC values as obtained from the graphic method are still useful in determining relative positions of certain members within the group being studied.

A survey of the literature reveals that no base compositions have been reported for the genus Actinomyces, nor for O. viscosus, Ramibacterium pleuriticum, Rothia dentocariosus, or C. haemolyticum. However, there have been values reported for some species which are now classified in the genus Actinomyces, and for members of Ramibacterium sp. and Corynebacterium sp.

Table 4. Base Composition of Representative Genera Related to the Actinomyces

Organism	%GC	Method
Lactobacillus parabifidus (Actinomyces parabifidus)	57.5*	chemical[a]
Proprionibacterium sp.	66.5-70.5*	chemical
Corynebacterium pyogenes	48.5*	chemical
Corynebacterium acnes	46-48**	physical (T_m)
Ramibacterium ramosum	30.0*	chemical

*Hill, 1966.

**Marmur, 1963.

[a]Hydrolysis followed by paper chromatography

Higher plants and animals possess DNA with approximately 38 ± 10 % GC. Several workers have speculated on the evolutionary significance of the wide range of microbial DNA base compositions. It would appear likely that there has been a gradual change in base compositions of bacterial DNA brought about as a result of mutations involving base changes.

Clearly, these data do not provide very fine species distinctions. Allowing for experimental deviation alone, the best that could be done was to place the organisms in one or the other of GC clusters. Differences in technique may help to explain the discrepancies between the GC content presented here for C. pyogenes and that reported in the literature. In addition, the small number of organisms whose DNA was satisfactorily isolated makes any generalizations inadvisable about the genus Actinomyces.

DNA Base Composition and Taxonomy of Actinomyces

Figures by Hill (1966) show that, whereas some genera are homogeneous in DNA base composition, others are very heterogeneous or even discontinuous. The latter finding is more useful taxonomically than the former, for DNA samples showing widely different base compositions can be assumed to have also different base sequences (i.e., genetic messages). On the other hand, it cannot be assumed that base sequences are similar in two DNA samples because of their overall base composition similarity. Therefore, in cases of genera showing homogeneity in DNA base composition, within a genus, the DNA data are not contradictory to the classification derived by other means. In those cases where there is heterogeneity in base composition among species of the same genus, the DNA data confirms the suspicion, often already aroused by standard taxonomic methods, that unrelated bacteria are being classified

together.

Figure 3 shows diagrammatically the ranges of some Gram-positive genera along with the ranges of the known and unknown Actinomyces spp. studied. In general, there has been good agreement between taxonomic conclusions based on numerical taxonomy and those based on GC content of DNA. The three Actinomyces spp. studied showed a range of 45-64% GC. A. proprionicus and A. discofoliatus do not differ by more than 10% GC and this confirms the numerical taxonomy putting them into the same genus. However, the inordinately high GC value for A. bovis cannot contradict its present classification, as stated above in the rules of base composition and taxonomy. The two Corynebacterium spp. which were analyzed fall relatively into the same GC range. This range also falls into the same range as the three Actinomyces spp., but the base composition cannot further classify the two species until further information is obtained.

Concerning the anaerobic diphtheroid groups prepared by Biever (1967), taxonomic conclusions can be drawn only on the organisms of each group from which DNA could be extracted. Fortunately, at least one organism from each group was characterized according to its base composition. These results are simply reported as an addendum to a composite table of groups proposed by Biever (1967), Urevig (1968), and Scheetz (1969) based on fermentation reactions, serology, and cell wall composition (Table 5).

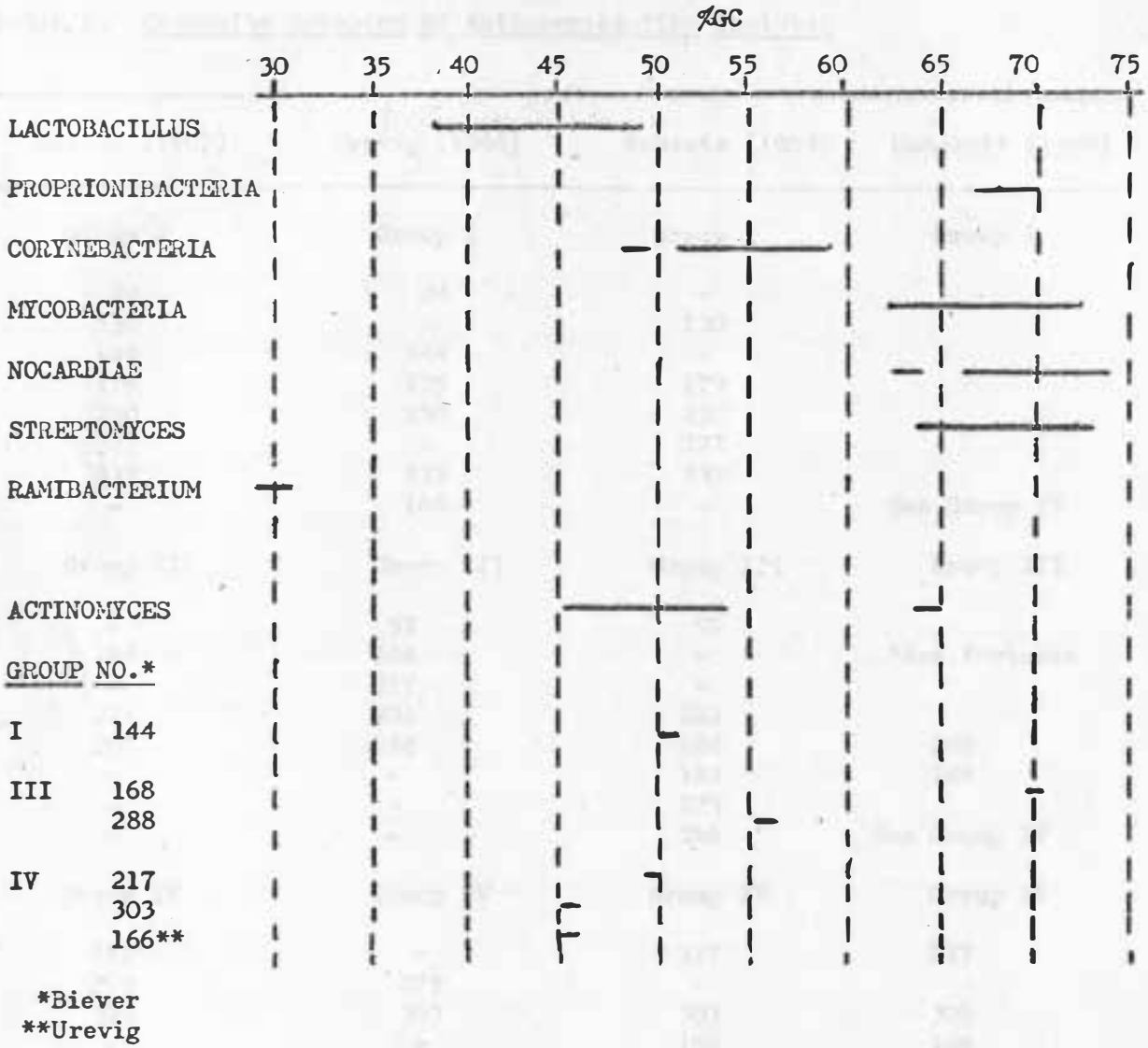

Figure 3. Relative Ranges of %GC Among the Actinomyces and Related Genera, Including Grouped Diphtheroids. (The ranges of the Actinomyces and grouped diphtheroids are taken from the graphic data of Table 3. The other genera are cited from Hill, 1966.)

Table 5. Composite Grouping of Actinomyces-like Isolates

Biever (1967)	Urevig (1968)	Scheetz (1969)	Campbell (1969)
Group I	**Group I**	**Group I**	**Group I**
24	24	-	
130	-	130	
144	144	-	
179	179	179	
230	230	230	
327	-	327	
333	333	333	
-	166	-	See Group IV
Group III	**Group III**	**Group III**	**Group III**
-	97	97	
168	168	-	*See footnote
-	217	-	
221	221	221	
288	288	288	288
-	-	144	144
-	-	279	
-	-	166	See Group IV
Group IV	**Group IV**	**Group IV**	**Group IV**
217	-	217	217
279	279	-	
303	303	303	303
-	-	168	166

*Isolate no. 168 did not fit into any of the groups.

FURTHER IMPLICATIONS AND SUGGESTED STUDIES

The ideal situation for classifying organisms would be one in which all of the properties of a particular organism are known. One approach would require a knowledge of all the phenotypic expressions or a significant cross-section of them. This is the basis of Adansonian analysis (numerical taxonomy). A more meaningful approach would be to study genetic compatibility of the organisms as well as the informational molecules (nucleic acids and proteins) which are ultimately responsible for the expression of the phenotypic traits. Thus, a more complete knowledge of the base compositions for each species of the Actinomyces would be needed; it would be wise to include those unknown isolates earlier postulated as being a new species, A. suis. This would entail initially a perfected method of obtaining a better yield of native DNA from each organism. The next point is to spend the necessary time in assaying enzymes and/or chemical agents which might effectively lyse those resistant cells. A consistent investigation of variations in extraction procedures should be carried out, rather than in a trial and error fashion.

When DNA can be consistently isolated in a purified, native form in good yield, thermal denaturation curves can be traced, with duplicates of different portions of the same sample and of different preparations of the same source. Several strains of the same organism are also necessary if one is to account for variations within a species. Then, a second method of determining base composition, preferably buoyant density, should be utilized to confirm T_m values. Buoyant density also increases linearly with increase in GC content. It is estimated in a cesium chloride density gradient and microdensitometer tracings are made of the resulting densities of the

sample and reference DNA's.

The average base composition for a series of organisms does not indicate any information about the base sequences. True phylogenetic relatedness among organisms can be confirmed only when base sequences are compared; this is done using genetic homologies. Two organisms possess extensive genetic homology if they are able to undergo syncytic recombination. However, a limited number of genetic determinants can be transferred from one organism to another by an autonomous episome whose DNA composition may differ appreciably from that of the recipient. Conveniently, the extent of genetic homology can better be assessed by measuring renaturation between single-stranded DNA preparations of diverse origin. Methods of obtaining genetic homologies include isotope labelling with subsequent cesium chloride gradient densities or agar-immobilization techniques.

CONCLUSIONS

(1) Certain of the *Actinomyces* spp. as well as a number of the *Actinomyces-like* isolates were successfully lysed by pronase, grinding and sodium lauryl sulfate to yield native DNA.

(2) Base ratios using the thermal denaturation method were obtained for those diphtheroids from which polymerized, native DNA was isolated.

(3) The GC content of the *Actinomyces* spp. fell within a range below that of the other members of the *Actinomycetales*, except for *A. bovis* which had a higher GC value than the other *Actinomyces* studied.

(4) The %GC as determined by the graphic method was about 20% lower than the %GC calculated from the theoretical equation of Schildkraut and Lifson, as cited by Hill, 1968.

(5) The GC content of the isolates from Groups I, III, and IV (Biever's serological grouping) ranged from 45-55 %, except for #168, which had 70 %GC.

(6) On the basis of GC content, isolate #166 was regrouped into Group IV, and #168 was not grouped into any of the existing groups.

(7) The %GC as determined from T_m values only indicates possible relationships among the diphtheroid isolates and the *Actinomyces* spp., but is not of itself valuable in reclassification of the organisms in this study.

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